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ANTIBACTERIAL ACTIVITY AND SCANNING ELECTRON MICROSCOPY (SEM) EXAMINATION OF ALGINATE-BASED FILMS AND WOUND DRESSINGS

WŁAŚCIWOŚCI ANTYBAKTERYJNE I SKANINGOWA MIKROSKOPIA ELEKTRONOWA (SEM) W BADANIACH FILMÓW ALGINIANOWYCH I OPATRUNKÓW AKTYWNYCH

Abstract: Natural polymers widely used to produce drug carriers and active dressings include alginates, gelatine, chitosan and hyaluronic acid. In this study, alginate films were obtained bypassing the process of lyophilization. Produced from the common bladder wrack (*Fucus vesiculosus* L.), they can be used in food chemistry, wound treatment (tissue infections, burns) and in skin care; they are also good drug carriers. The films were examined for their bacteriostatic effects. Alginate gels exhibit bacteriostatic properties against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*. Tests conducted for active dressings revealed that Aquacel Ag and gauze soaked with 1% AgNO₃ exhibit bacteriostatic properties against *E. coli* and no resistance against *S. aureus*. Examination using scanning electron microscopy (SEM) confirmed the bacteriostatic properties of the Aquacel Ag dressing and gauze with 1% AgNO₃. Dead cultures of *Staphylococcus aureus* were observed on the fibre surface of both dressings.

Keywords: antibacterial, scanning electron microscopy, alginate films, wound dressings

Alginate, a polysaccharide extracted from marine brown algae (*Phaeophyceae*), is a common type of gelling agent employed in the food industry [1, 2]. It is of interest as a potential film or coating component because of its unique colloidal properties. These include thickening, stabilizing, suspending, film forming, gel producing and emulsion stabilizing properties [3, 4].

Microbial growth on food surfaces is a major cause of food spoilage. In particular, bacterial contamination of ready-to-eat products is of serious concern to human health. Antimicrobial agents used in food application include organic acids, bacteriocins, enzymes, alcohols and fatty acids [5]. The beneficial effects obtained by using edible film and coating in terms of physical, mechanical and biochemical benefits have been reported [6]. Edible

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films can improve shelf life and food quality by serving as selective barriers to moisture transfer, oxygen uptake, lipid oxidation and losses of volatile aromas and flavours [7, 8]. Their use is gaining importance in food protection and preservation as they provide advantages compared with films made from synthetic materials [9]. Addition of hydrocolloids such as alginate may improve the barrier and tensile properties of fruit-based films [1].

Biopolymers from marine sources have also been studied and utilized in pharmaceutical and biotechnological products. Carrageenans are watersoluble galactose polymers extracted from red seaweed, which are extensively used in food and pharmaceutical industries as gelling and stabilizing agents. Carrageenan has one negative charge per disaccharide with a tendency to form excellent gel and film forming properties, and exhibits the highest tensile strength. Alginic acid is a copolysaccharide extracted from brown algae consisting of D-mannuronic and L-guluronic acid monomers. The ability of alginates to react with di- and trivalent cations is being utilized in alginate film formation. Na-alginate is a water soluble salt of alginic acid, a naturally occurring non-toxic polysaccharide. With regard to the film forming properties of Na-alginate and k-carrageenan, numerous controlled or sustained-delivery systems have been described in literature [10-13] whereas the activity of these biopolymers as antimicrobial agents in such films has not been thoroughly researched.

Many modern wound dressings have a variety of properties that are designed to create an environment to encourage conditions that support wound healing. These include the ability to absorb exudate, provide optimum moisture balance at the wound surface and prevent maceration of surrounding tissue. Since bacteria are often present in high numbers in wound fluid, it is also important that dressings with high fluid retention levels be able to absorb and retain bacteria. Once the skin barrier is broken, there is a much greater risk for infection as the majority of wounds provide a favourable environment for both aerobic and anaerobic bacteria [14]. It is self-evident that if a wound is bacteria-free then infection cannot occur. The prevention of wound infection and a reduction in cross-infection of wound pathogens are primary concerns in infection control.

Lawrence [15] demonstrated that the dispersal of bacteria was reduced by 20% when a moisture retentive hydrocolloid was removed from a simulated wound, compared with a gauze dressing. If a wound remains unhealed for more than six weeks, it becomes chronic and in these cases complications become more frequent because of bacterial colonization [14]. Some of these dressings are medicated, containing active substances to manage the microbial load in the area of the wound. Typical actives include antibiotics (such as neomycin, bacitracin or polymyxin combinations) and broad-spectrum germicidal agents (silver, iodine, chlorhexidine, etc.).

Silver in its numerous forms, including metallic silver, silver nitrate, silver sulfadiazine and silver calcium phosphate, has been used for over 200 years in the treatment of burn injury [16]. It is effective against a broad range of aerobic, anaerobic, Gram-negative and Gram-positive bacteria, especially *Staphylococcus aureus*, *S. epidermidis* and *Klebsiella pneumoniae*, as well as yeast, filamentous fungi and viruses [17]. Silver nitrate has been used historically as an antiseptic agent. In concentrated form, it is highly toxic to tissues. However, an aqueous solution of 0.5% silver nitrate offers significant antimicrobial activity without tissue toxicity.

It is very important to investigate the possibility of producing antimicrobial alginate film by incorporation of AgNO_3 and H_4SiO_4 . The objective of this study was to assess the

antibacterial activity of alginate films and wound dressings against the pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*.

Experimental

Materials

Film preparation

Films were extracted from the common bladder wrack (*Fucus vesiculosus* L.), which belongs to marine brown algae. Weeds from two producers were used for examination, packed and distributed by the Zakład Konfekcjonowania Ziół Flos, Morsko, Poland and by the Witherba S.C. Zioła, Piotrków Trybunalski, Poland.

Dried *Fucus vesiculosus* L. was dipped in an aqueous formaldehyde solution (3.7%, 100 cm³) and kept in a closed flask at 30°C overnight to remove lipophilic compounds. The suspension was stirred and extracted at 80°C for 4 hours with 0.1 M HCl (100 cm³), 1 M HCl (100 cm³), 1% HCl (100 cm³) or 0.085 M Na₂CO₃. Then it was filtered through the Büchner funnel. The filtrate was vacuum-condensed to about ¼ volume. During and just before the end of condensation, the solution was rinsed with a small amount of 96% ethanol. The gelling precipitate was poured onto Petri plates. Some films were poured over with 10% CaCl₂, taken out and dried. All films were dried overnight on the plates at 25°C or 50°C.

Also examined were 100% sodium carboxymethylcellulose commercial active dressings: Aquacel (ConvaTec) and Aquacel Ag (ConvaTec), as well as cotton or viscose gauze soaked with 1% AgNO₃ or 1% H₄SiO₄.

Antimicrobial treatment

Two kinds of alginate gels, produced from the common bladder wrack by Witherba and Flos, were examined. They were exposed to bacteria that can cause nosocomial infections, that is the Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*.

Physiological salt (2 cm³) was poured into two sterile test tubes. Using a sterile (red hot) inoculation loop, *E. coli* sample was taken from its culture on enriched agar (used for growing particularly demanding bacteria strains), inserted into one of the test tubes and diluted in the salt. Using a pipette, 3 drops of the suspension were transferred onto enriched agar; then, using a cooled sterile bacteria spreader, they were spread all over the agar surface. After that, the spreader was sterilized again and 3 drops of alginate gel were placed in the centre of the Petri plate, using a dropper. The same procedure was repeated for *Staphylococcus aureus*, which was placed on mannitol salt agar (containing 7.5% NaCl for inhibiting growth of other bacteria). The Petri plates were subsequently placed in a tube and then kept in a laboratory heater at 37°C for 24 h and 48 h.

Dressing materials were exposed to the same bacteria as the alginate gels: *Escherichia coli* on MacConkey agar (containing salts of bile acids and crystal violet inhibiting growth of Gram-positive bacteria) and *Staphylococcus aureus* on mannitol salt agar (with high concentration of NaCl inhibiting growth of other bacteria). The samples were kept in a laboratory heater at 37°C for 24 h, and then photos were taken.

Scanning electron microscopy (SEM)

Fibre surface was also examined using a JSM 5500LV scanning electron microscope supplied by JEOL. Secondary electrons (SE) and back-scattered electrons (BSE) observations were conducted, with the accelerating voltage of 5 kV. Microphotographs were taken at magnifications ranging from 500× to 10,000×.

Results and discussion

The objective of this study was to produce from the common bladder wrack (*Fucus vesiculosus* L.), bypassing the process of lyophilization, a series of alginate films and to select a gel exhibiting good antibacterial effects. Figure 1 shows some examples of such films.



Fig. 1. Examples of alginate films: 1.5% alginic acid gel (a); non-defatted alginate film (b); defatted alginate film (c and d)

Antimicrobial activity of alginate films

Corrected zone of inhibition tests were used to determine the antimicrobial activity of the alginate films. They proved that both Flos and Witherba gels inhibit growth of Gram-negative *Escherichia coli*. There were no inhibition zones in samples of

Staphylococcus aureus. Results of these observations are shown in Table 1 and, as an example, Figures 2 and 3.

Antibacterial activity of alginate films

Table 1

Samples	Bacteria	Inhibitory zone
Flos	<i>Escherichia coli</i>	+
Flos	<i>Staphylococcus aureus</i>	-
Witherba	<i>Escherichia coli</i>	+
Witherba	<i>Staphylococcus aureus</i>	-

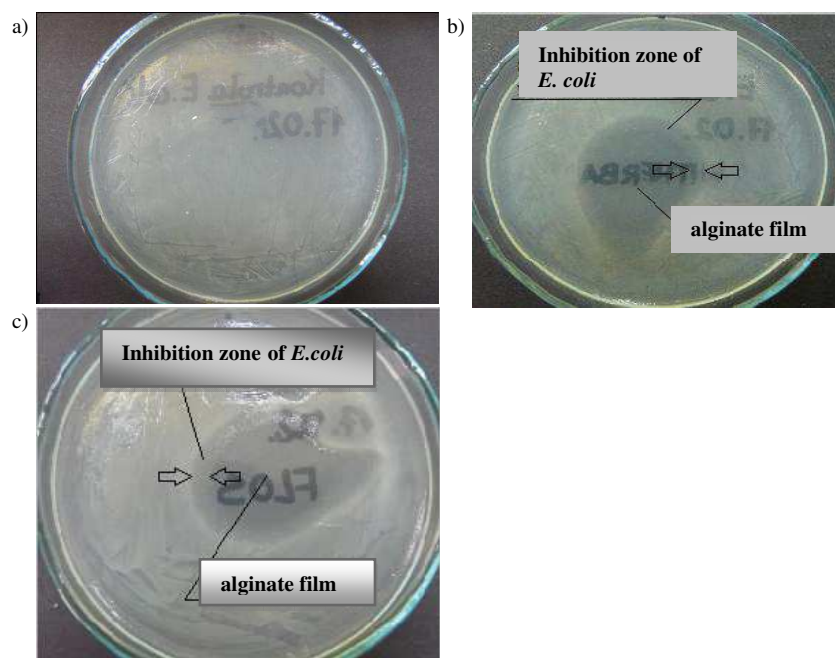


Fig. 2. Inhibition zone of Witherba (b) and Flos (c) alginate films compared with reference (a) against *Escherichia coli* (24 h incubation)

Antibacterial activity of wound dressings

Table 2

Samples	Bacteria	Inhibitory zone
Aquacel (1)	<i>Escherichia coli</i>	-
Aquacel (1)	<i>Staphylococcus aureus</i>	-
Aquacel Ag (2)	<i>Escherichia coli</i>	+
Aquacel Ag (2)	<i>Staphylococcus aureus</i>	+
Gauze + 1% H ₄ SiO ₄ (3)	<i>Escherichia coli</i>	-
Gauze + 1% H ₄ SiO ₄ (3)	<i>Staphylococcus aureus</i>	-
Gauze + 1% AgNO ₃ (4)	<i>Escherichia coli</i>	+
Gauze + 1% AgNO ₃ (4)	<i>Staphylococcus aureus</i>	+

A parallel examination of commercial active dressings was also conducted in order to compare the results with those of examining the antibacterial activity of the alginate films. Both results are shown in Table 2 and Figure 4.

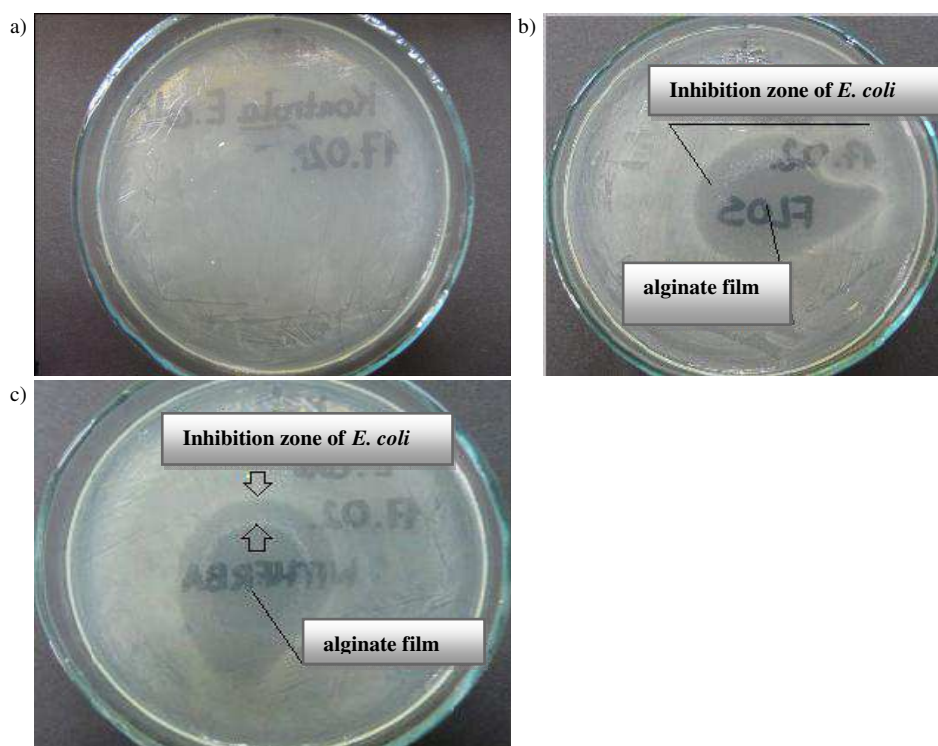


Fig. 3. Inhibition zone of Flos (b) and Witherba (c) alginate films compared with reference (a) against *Escherichia coli* (48h incubation)

The antibacterial tests proved that Aquacel Ag and gauze soaked with 1% AgNO_3 inhibit Gram-negative *Escherichia coli*. Inhibition zones appeared along the edges of the dressings. For neither Aquacel Ag nor gauze soaked with 1% AgNO_3 , there were any such zones against *Staphylococcus aureus*.

Silver has found particular application in medicated wound dressings as it shows broad antimicrobial (against both Gram-negative and Gram-positive organisms) and anti-fungal activity [18]. It has been suggested that alginate wound dressings may immobilize bacteria within their fibrous matrix. Bowler et al [19] have demonstrated that phenomena whereby a hydrated carboxymethylcellulose (AQUACELs Hydrofibers) wound dressing immobilized exudate containing bacterial populations within its cohesive gel structure. The composition, the arrangement of fibres, the fibre density and the relative proportion of individual groups (for example, guluronic and mannuronic acid groups which form fibres in alginate wound dressings) are important considerations and can influence the ability of a dressing to handle large volumes of exudate by absorbing and retaining microorganisms.

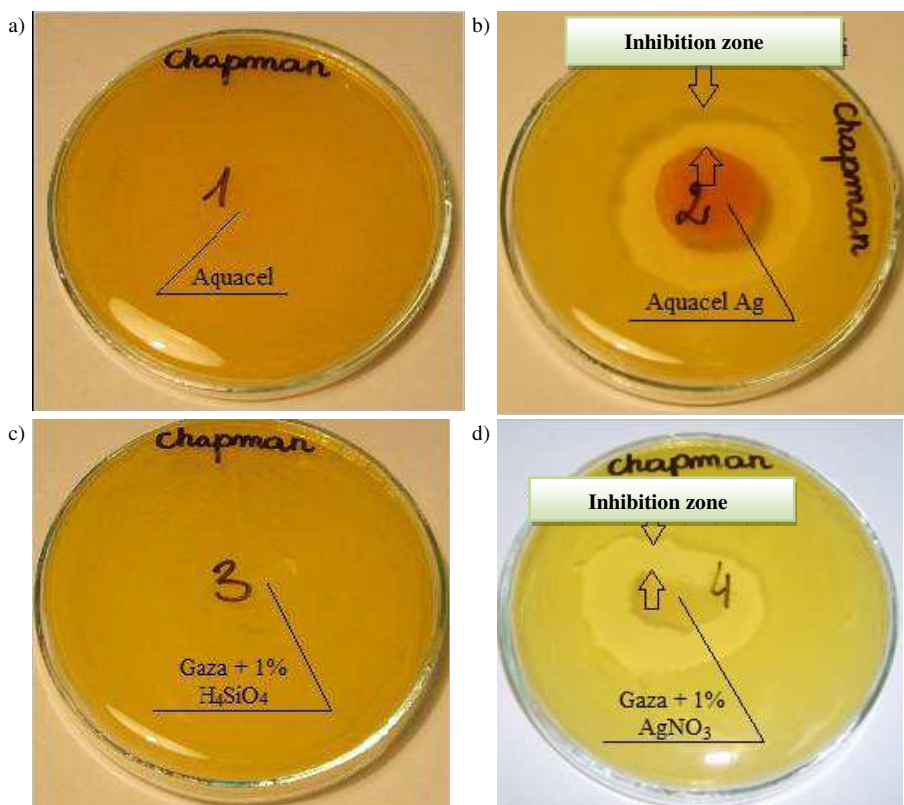


Fig. 4. Inhibition zone of Aquacel Ag (2) and gauze + 1% AgNO_3 (4) compared with Aquacel reference (1) and gauze + 1% H_4SiO_4 (3) against *Escherichia coli*

Several proposals have been developed to explain the inhibitory effects of Ag^+ ions on bacteria. In aqueous environments, silver ions are released and antimicrobial activity depends on the intracellular accumulation of their low concentrations. The ions avidly bind to negatively charged components in proteins and nucleic acids, thereby effecting structural changes in bacterial cell walls, membranes and nucleic acids that affect viability. In particular, silver ions are thought to interact with thiol groups, carboxylates, phosphates, hydroxyls, imidazoles, indoles and amines, either singly or in combination, so that multiple deleterious events rather than specific lesions simultaneously interfere with microbial processes [20]. Microbiological and chemical experiments imply that the interaction of Ag^+ ions with thiol groups plays an essential role in bacterial inactivation [21].

Perhaps the most unique form of silver developed for wound dressings is nanocrystalline silver, which differs in both physical and chemical properties from micro- or macrocrystalline silver and from silver salts. A unique property of nanocrystalline silver is that, according to Fan and Bard [22], it dissolves to release Ag^0 clusters and Ag^+ , whereas other silver sources release only Ag^+ . This difference in the dissolution properties of nanocrystalline silver dressings appears to alter the biological properties of the solution, including both antimicrobial and anti-inflammatory activity. Nanocrystalline silver

dressings have been demonstrated in vitro as effective antifungal agents, antibacterial agents [23] and antibacterial agents for antibiotic-resistant bacteria [24]. In vivo studies have shown that nanocrystalline silver is very effective at preventing infections and healing wounds [25].

Cotton and viscose gauze, although still much in use for acute wounds, has largely been replaced for the treatment of chronic wounds by modern wound dressings produced from alginate and, more recently, fibres made from carboxymethylated cellulose (NaCMC fibre, Hydrofibers, Aquacel ConvaTec Ltd). *Sodium carboxymethylcellulose* (NaCMC) fulfils these criteria, forming a soft gel or viscous solution with wound fluid. This gel-forming property observed for NaCMC has led to its use for wound care, in gel formulations and in hydrocolloid dressings that contain NaCMC in an insoluble matrix. Fibrous dressings made from alginate combine the properties of fluid absorbency and gel formation and some also retain their integrity so that they can be readily removed. The presence of the methyl carboxyl group ($-\text{CH}_2\text{COO}^-$) in carboxymethyl gauze permits further chemical modification by partial cation exchange of sodium by silver to develop a post-treated antimicrobial product [26]. Silver cations are microcidal at low concentrations and are used to treat burns wounds and ulcers. These new dressings contain various forms of silver, ranging from pure metallic silver (Silverlon) to compounds such as silver carboxymethylcellulose (Aquacel Ag), silver phosphate (Arglaes) and silver chloride (Silvasorb) [27].

Scanning electron microscopy (SEM)

Scanning electron microscopy has demonstrated the immobilization of bacteria within Hydrofibers [28]. Therefore, the aim of this study was to develop a suitable SEM technique that would allow for the visualization of bacteria within fibrous wound dressings.

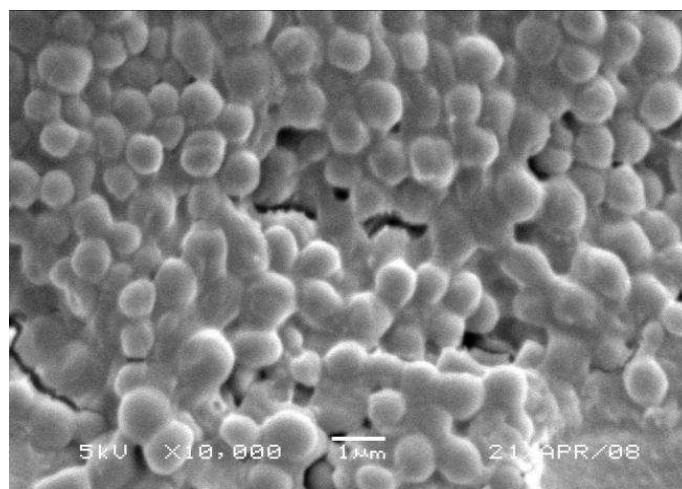


Fig. 5. *Staphylococcus aureus* adhering to the surface of Aquacel fibres

A key challenge for maximizing the healing response in wounds has been the advent of modern fibrous dressings to absorb large volumes of exudate, while still providing moisture

balance in the wound environment [29]. Equally important, however, is the ability of these dressings to immobilize wound exudate, which may contain pathogenic bacteria (such as *P. aeruginosa* or *Staphylococcus aureus*). Bowler et al [19] have suggested that as Hydrofibers dressings absorb wound exudate, this in turn reduces the interstitial spaces between individual fibres within the dressing as they coalesce, resulting in bacterial immobilization. Subsequent studies using SEM have confirmed these findings [28].

In this study, since there were no inhibition zones against *S. aureus* in any of the samples examined, fibre surface was investigated using SEM. The results are shown in Figures 5-7. In Figure 6, *S. aureus* cultures fill the interstitial spaces between individual fibres of the Aquacel dressing and grow on their surface.

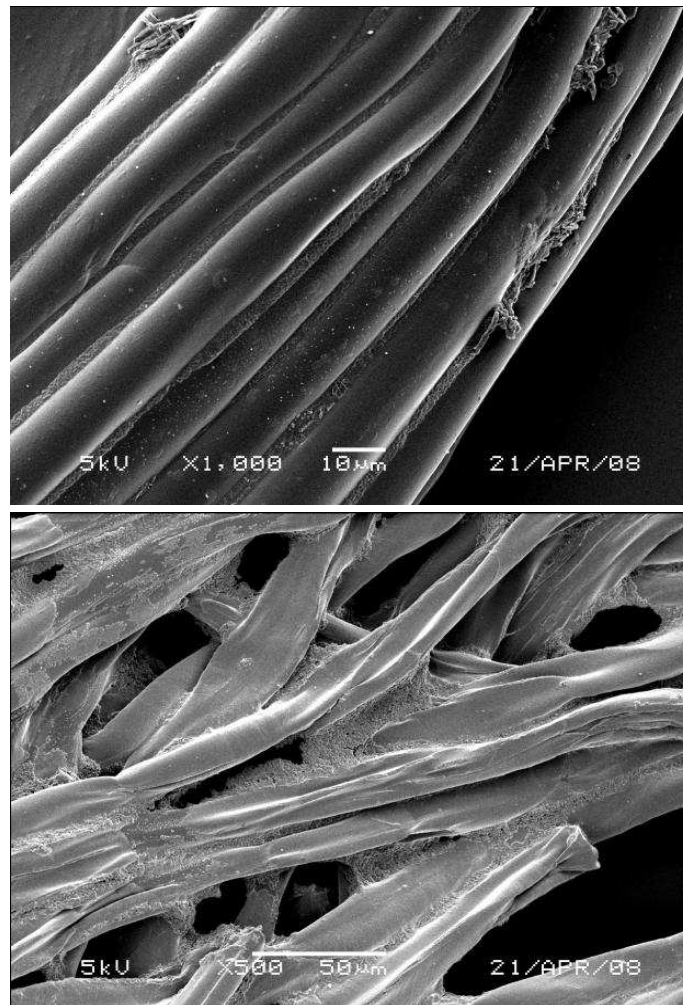


Fig. 6. Bacteria adhering to the surface of Aquacel fibres

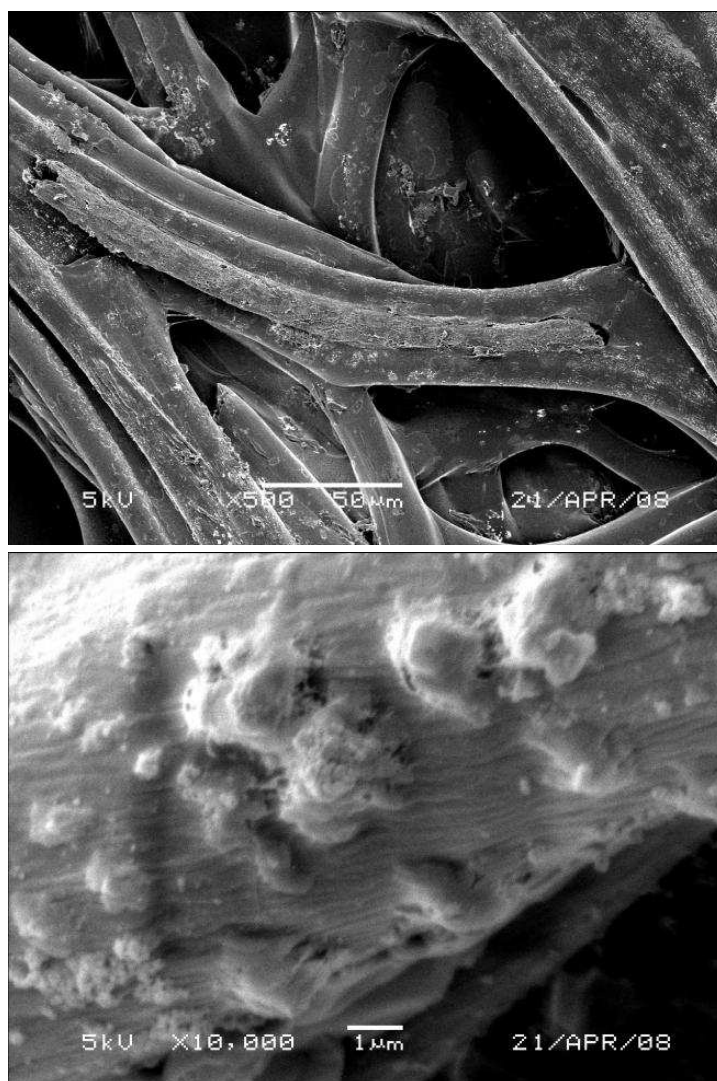


Fig. 7. Bacteria adhering to the surface of and filling the spaces between Aquacel fibres

Figure 7 proves that the Aquacel Ag dressing is effective against Gram-positive bacteria (as it releases bioactive Ag^+ cations). The amount of the bacteria growing on the fibre surface is substantially lower and degraded cultures of *Staphylococcus aureus* can also be seen. One possible explanation for this could be related to the presence of a thinner cell wall in Gram-negative bacteria compared with the much thicker and more complex cell wall present in Gram-positive bacteria. Feng and Kim [30] have suggested that the presence of the thicker cell walls “is of immense practical importance in protecting the cell from penetration of silver ions into the cytoplasm”. Studies are under way to examine the effects of ionic silver on the bacterial cell wall. Generally, the bacteria closest to the Aquacel Ag

hydrating fibres interact with available silver ions first and the bactericidal effect continues as bacterial suspensions move along the fibres throughout the extended experimental periods (Fig. 7).

Earlier it has been demonstrated that both the alginate films produced for this study and the commercial active dressings of the Aquacel Ag type, as well as gauze soaked with a solution of silver nitrate, exhibit bacteriostatic properties. The results of these examinations are shown in Figures 2-4. Bacteriostatic effects of gauze soaked with 1% AgNO_3 and H_4SiO_4 were also observed. The results are shown in Figures 8-10.

Gauze soaked with 1% H_4SiO_4 (Fig. 8) reveals the presence of *Staphylococcus aureus* growing on its surface while gauze soaked with 1% AgNO_3 (Fig. 9) is more inhibitory to this growth. Gauze soaked with 1% AgNO_3 (Fig. 10) exhibits, in the same way as the Aquacel Ag dressing, bacteriostatic properties, as confirmed by the dead bacteria cultures.

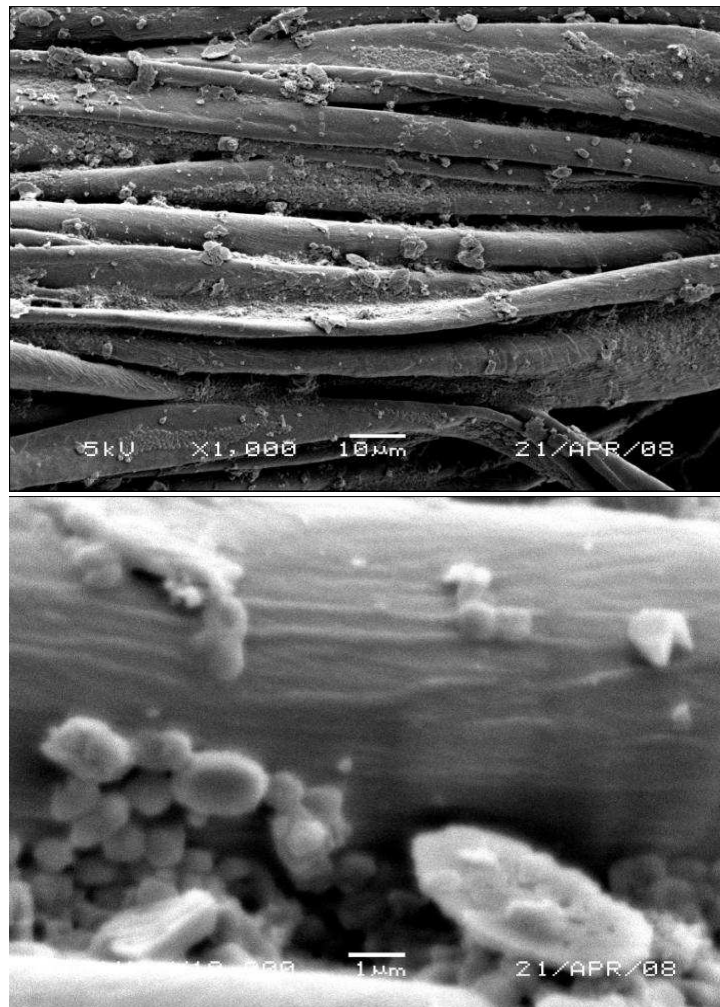


Fig. 8. Bacteria adhering to the surface of cotton gauze fibres

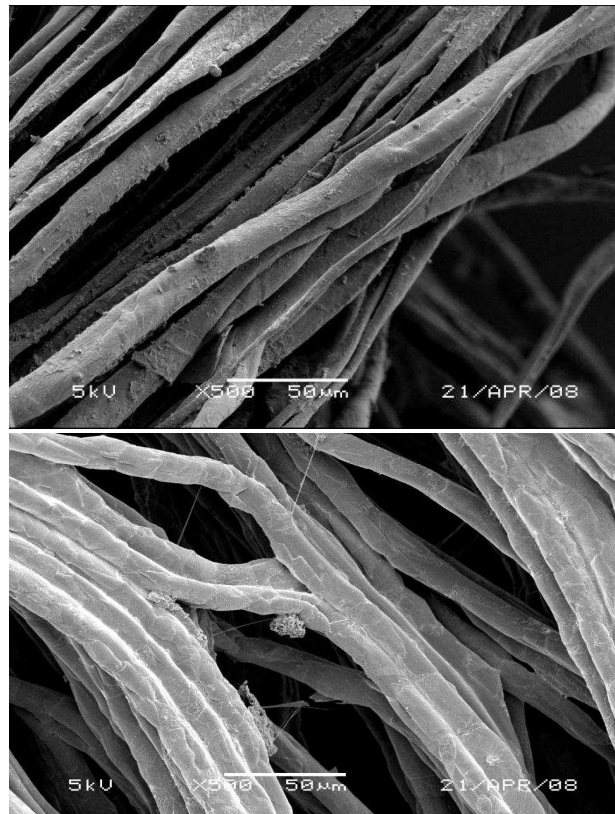


Fig. 9. Bacteria adhering to the surface of cotton gauze fibres

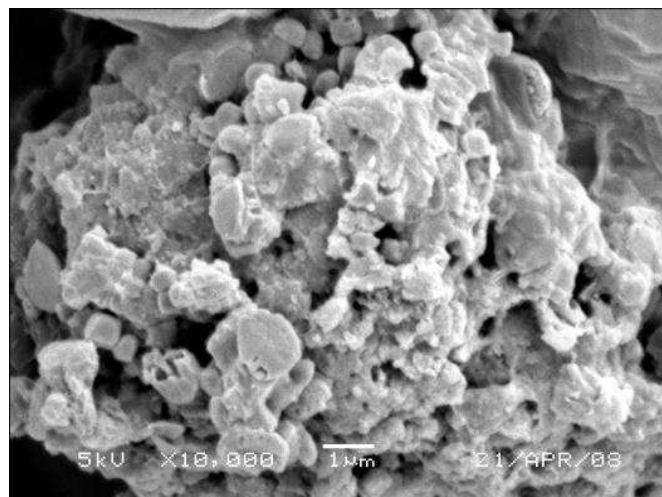


Fig. 10. Bacteria adhering to the surface of cotton gauze fibres

To summarize these findings, it should be said that the alginate films exhibit bacteriostatic properties against Gram-negative bacteria *Escherichia coli* and no resistance against Gram-positive *Staphylococcus aureus*. Tests with inhibitory spaces conducted for active dressings show that Aquacel Ag and gauze soaked with 1% AgNO₃ have bacteriostatic effects against *E. coli* and no resistance against *Staphylococcus aureus*. Compared with alginate gels, Aquacel Ag and gauze soaked with 1% AgNO₃ offer more effective antimicrobial activity, as demonstrated by larger inhibition zones around the dressings. SEM examination has confirmed the antibacterial properties of the Aquacel Ag dressing and gauze soaked with 1% AgNO₃. Dead cultures of *Staphylococcus aureus* have been observed on the surface of both dressings. Aquacel and gauze soaked with 1% H₄SiO₄ have proved to offer no resistance against *E. coli* and *S. aureus*.

Conclusions

The alginate films/gels produced from the common bladder wrack (*Fucus vesiculosus* L.), bypassing the costly process of lyophilization, exhibit good antibacterial effects. These gels can be used in food chemistry, pharmacy, medicine and cosmetology along with the widely produced active dressings.

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WŁAŚCIWOŚCI ANTYBAKTERYJNE I SKANINGOWA MIKROSKOPIA ELEKTRONOWA (SEM) W BADANIACH FILMÓW ALGINIANOWYCH I OPATRUNKÓW AKTYWNYCH

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Abstrakt: Naturalne polimery są szeroko używane do opatrunków aktywnych na bazie: alginianów, żelatyny, chitozanu i kwasu hialuronowego. W przedstawionych badaniach błonę alginianową otrzymuje się, omijając liofilizację. Żel otrzymano z morskoczynu pęcherzykowatego (*Fucus vesiculosus* L.) wykorzystywanego powszechnie w chemii żywności, leczeniu ran (zakażeniach tkanek, oparzeniach), pielęgnacji skóry oraz jako nośnik leków. Badane żele alginianowe wykazywały właściwości bakteriostatyczne w stosunku do bakterii Gram-dodatnich *Staphylococcus aureus* oraz bakterii Gram-ujemnych *Escherichia coli*. Testy przeprowadzone dla aktywnych opatrunków wykazały, że Aquacel Ag i gaza nasączona 1% AgNO₃ wykazują właściwości bakteriostatyczne w stosunku do *S. aureus* i *E. coli*. Badanie za pomocą skaningowego mikroskopu elektronowego (SEM) potwierdziło właściwości bakteriostatyczne Aquacel Ag i opatrunku z gazy nasączonym 1% AgNO₃. Na powierzchni włókien obu opatrunków zidentyfikowano obumarłe kultury *S. aureus* i *E. coli*.

Słowa kluczowe: właściwości antybakteryjne, skaningowy mikroskop elektronowy, błona alginianowa, opatrunki